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14. ABSTRACT In this funded research, we have described "zfration" - a naturally occurring small-size peptide Zfra undergoes self-covalent binding and aggregation, and may covalently interact with tumor suppressors for functional inactivation. Zfra is a 31-amino-acid peptide, whose composition is similar to that of zinc finger family proteins. In the original Specific Aims, we proposed to 1) determine how Zfra undergoes covalent self-aggregation and binding with tumor suppressor proteins for regulating their functions; 2) determine the overall protein targets with which Zfra interact; 3) investigate the effect of Zfra deficiency in breast cancer cells. As research ongoing as planned, we have achieved excellent progress and determined that Zfra can 1) induce degradation of intracellular zfrated proteins, 2) undergo enzyme-independent self-polymerization for more than 200 fold of its original size, and 3) significantly increase the growth of breast and skin basal cell carcinoma in nude mice by 3-4 fold in 3 months, suggestive of its inactivation of tumor suppressors <i>in vivo</i> .				
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Introduction

Our discovery of tumor suppressor WWOX/FOX1 and inhibitor Zfra – In year 2000, we and two other groups have independently discovered a candidate tumor suppressor, named WW domain-containing oxidoreductase (designated WWOX, FOR, or FOX1) (1-5). Up to date, PubMed database shows that there are more than 130 publications regarding WWOX/FOX1, indicating that this protein is a hot target for cancer research. Human WWOX gene is mapped to a common fragile site on chromosome ch16q23.3-24.1 (1,2). Approximately 30-50% of loss of heterozygosity (LOH) of human WWOX gene has been found in cancers in breast, prostate, and many other organs. WWOX/FOX1 possesses a nuclear localization sequence (NLS), two N-terminal WW domains (containing conserved tryptophan residues) and a C-terminal short-chain alcohol dehydrogenase/reductase (SDR) domain (Fig. 1). At least 8 splice variants of human WWOX mRNA, coding for the altered C-terminal SDR domain sequences, have been found in cells. Over the past 10 years of research, a clear **big picture** for WWOX/FOX1 is its participation in **1)** apoptotic and stress responses *in vivo* and *in vitro* (3,6-12), **2)** regulation of tumor development *in vivo* (1-5,10,13-27), **3)** coordination in signaling and regulation of gene transcription (3,4,6,7,12-18,28,29), and **4)** neural development, degeneration and injury and organogenesis *in vivo* (8,9,21,27,30-32).

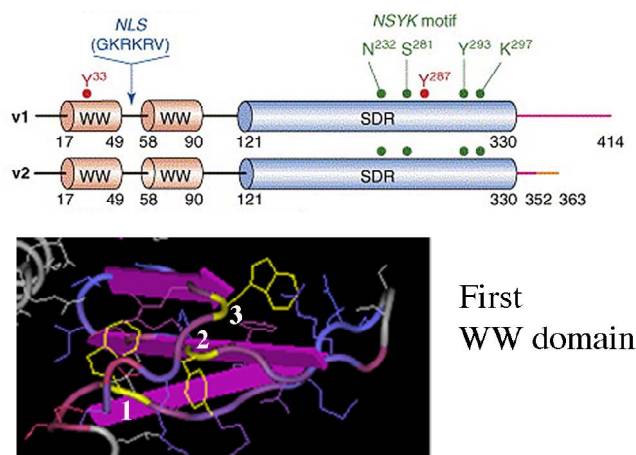


Fig. 1. WWOX/FOX1 and splice variants. The predicted amino acid sequence (414 amino acids) of FOX1 (WWOX or FOR) possesses two N-terminal WW domains, a nuclear localization signal sequence (NLS), and a C-terminal short-chain alcohol dehydrogenase (SDR) domain. A mitochondria-targeting sequence in FOX1 was mapped within the SDR domain (amino acid #209-273) (1-4). The structure of isoform FOX2 (WWOXv2; 41 kDa) is also depicted. The C-terminal amino acid sequence of FOX2 is different from FOX1 (in yellow). A simulated structure of the first WW domain is shown. 1= 1st tryptophan; 2= Try33 phosphorylation site; 3= 2nd tryptophan. (These residues marked in yellow) (adapted and modified from ref. #4)

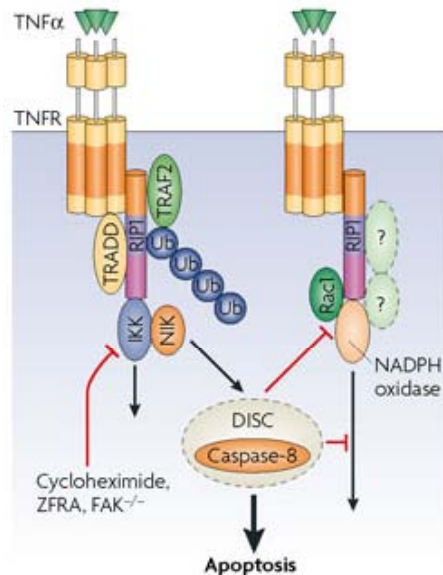
Our laboratory was the first to discover the proapoptotic function of FOX1 *in vitro* and *in vivo* (3,6-12). Under stress conditions, FOX1 becomes “activated” via phosphorylation at Tyr33 and is accumulated the mitochondria and nuclei to induce apoptosis in cultured cells and in the neural system of animals, as verified by confocal and immunoelectron microscopy (3,6-12) (Fig. 2). FOX1 binds numerous proteins in the stress signaling and apoptotic responses (3,4,6-12), as well as in transcriptional regulation (Fig. 2) (11-18), whereas the FOX1 signaling network and its physiological relevance remains elusive.

Role in tumor suppression and morphogenesis – It is not surprising to find that a tumor suppressor(s) is essential for cell differentiation, organogenesis and survival of an organism. One of the examples is tumor suppressor p53 (e.g. role in aging). Whole body *Wwox* gene ablation in mice (*Wwox*^{-/-}) have recently been developed (19). *Wwox*^{-/-} mice spontaneously developed several types of tumors, but they can only survive for approximately one month (19). By gene-trap targeting, homozygous *Wwox* gene-trap mice (*Wwox*(gt/gt)) were “hypomorphic”, as these mice have minor amounts of *Wwox*/FOX1

protein in several tissues (20). These hypomorphic mice had a longer life span, and female hypomorphs had a higher incidence of spontaneous B-cell lymphomas. Testes from *Wwox* (gt/gt) males had high numbers of atrophic seminiferous tubules and reduced fertility when compared with wild-type counterparts, suggesting that murine *Wwox* gene is involved in the development and differentiation of male reproductive organs. An additional study also revealed that *Wwox* gene is essential for postnatal survival in mice (21).

Fig. 2. Signaling network of WWOX/WOX1 and modes of actions. **Route 1.** Stress-induced activation of WWOX involves Tyr33 phosphorylation. Tyrosine kinase Src phosphorylates WWOX at Tyr33. Phosphorylated WWOX (p-WWOX) translocates to the mitochondria or nuclei *in vitro* and *in vivo*. p-WWOX may bind activation p53 with Ser46 phosphorylation, and the complex relocates to the mitochondria (4; review). WWOX stabilizes p53 and both proteins induce apoptosis in a synergistic manner (3,4,6,7). (For other Routes, see ref. #4)

Zfra is a negative regulator of WOX1 and p53 – To investigate functional inactivation of WOX1 by cellular proteins, we isolated Zfra from yeast two-hybrid cDNA library screen (33). Zfra is a 31-amino-acid zinc finger-like protein. We demonstrated that Zfra regulates cell death in the pathway of tumor necrosis factor (TNF) by physically interacting with downstream adaptors TNF receptor- or Fas-associated death domain proteins (TRADD and FADD) (Fig. 3) (11,12,34,35). Remarkably, Zfra inhibits Bcl-2/Bcl-xL expression and blocks cytochrome c release from the mitochondria, and yet induces apoptosis when overexpressed (12). Interestingly, Zfra undergoes self-association in response to stress stimuli (33). Zfra interacts with TRADD, NF-κB, JNK1, and WOX1 (11). Transiently overexpressed Zfra sequesters NF-κB (p65), WOX1, p53 and phospho-ERK (extracellular signal-activated kinase) in the cytoplasm (11). TNF or UV light could not effectively induce nuclear translocation of these proteins. Importantly, Zfra blocks the apoptotic function of activated Tyr33-phosphorylated WOX1 and Ser46-phosphorylated p53 (11).



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Fig. 3. Zfra in TNF signaling. Activation of apoptosis as a primary response to TNF requires specific apoptosis-promoting conditions, such as the presence of protein synthesis inhibitors (cycloheximide), the overexpression of zinc finger-like protein (Zfra) polypeptide or a deficiency in focal adhesion kinase (FAK) kinase signaling. This leads to efficient pro-apoptotic death-inducing-signaling complex (DISC) formation and caspase-8 activation (34). (reviewed by Degterev A, Yuan J; adapted from *Nat Rev Mol Cell Biol.* **9**, 378-90, 2008)

Body

Ser8 participates in overexpressed Zfra-induced apoptosis – Zfra is a 31-amino-acid protein possessing 5 conserved phosphorylation sites, including Ser2, Ser3, Ser8 and Thr20 for protein kinase C and Ser7 for cAMP- and cGMP-dependent protein kinase, respectively (33). Not all cells are sensitive to Zfra-mediated cell death. We have tested a panel of cancer cell lines derived from breast, brain, prostate, lymphoid tissue and fibroblasts, and found that prostate DU145 and mink lung epithelial Mv1Lu cells are resistant to Zfra-mediated cell death (11,33). Alteration of Ser8 to Gly8 abolishes the apoptotic function of Zfra (Fig. 4), suggesting that phosphorylation of Ser8 is essential for overexpressed Zfra to induce apoptosis. However, alteration of Thr20 to Gly (T20G) did not affect the death-inducing activity of Zfra (Fig. 4). Zfra-mediated apoptosis involves appearance of flip-flopped phosphatidylserine on the cell surface, nuclear condensation and internucleosomal DNA fragmentation (11,12,33).

Ser8 is a phosphorylation site in Zfra – We determined whether Ser8 undergoes phosphorylation in Zfra. We generated antibody against a synthetic Zfra peptide with Ser8 phosphorylation (designated pS8Zfra; 15 amino acid residues from the N-terminus). The antibody was affinity purified as described (6). COS7 fibroblasts were exposed to UV light, followed by processing immunofluorescence microscopy using our generated specific antibodies. UV light increased the expression of Zfra and its phosphorylation at Ser8 (Fig. 5). Also, there was an increased nuclear accumulation of Zfra (Fig. 5), indicating its relocation to the nuclei from the cytoplasm.

Under similar experimental conditions, UV light upregulated Zfra protein expression and Ser8 phosphorylation, as determined by Western blotting (Fig. 6A). A full-length Zfra peptide was synthesized and mixed with the cytosolic preparations of COS7 cells and incubated at 37°C. The results showed that there was a rapid upregulation of Zfra phosphorylation at Ser8, followed by dephosphorylation and degradation of the peptide (Fig. 6B). The observations suggest the presence of

specific enzyme(s) that interacts with Zfra for degradation. Alternatively, Zfra is small in size and may act like a protein modifier to enhance degradation of target protein and itself, an action similar to that of ubiquitin (36).

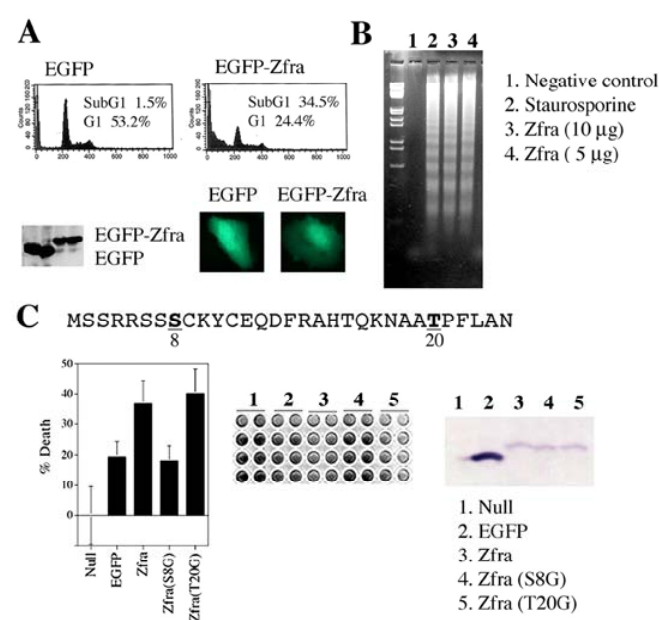


Fig. 4. Ser8 but not Thr20 is critical for Zfra-induced cell death. (A) L929 cells were electroporated with the cDNA expression construct of EGFP or EGFP-Zfra, followed by culturing for 48 hr. Compared to the EGFP controls, EGFP-Zfra induced cell death, as evidenced by a significant reduction in the G1 phase and an increase in the subG1 phase of the cell cycle (analyzed by FACS). (B) Zfra-induced internucleosomal DNA fragmentation in L929 cells is shown. In positive controls, cells were treated with staurosporine (1 µM) for 8 hr. In negative controls, cells were transfected with the EGFP vector only (10 µg). One set of data is presented from 3 experiments. (C) Neuroblastoma SK-N-SH cells were electroporated with the above constructs or medium (null) only. The cells were cultured 24 and 48 hr, and then stained with crystal violet. Compared to wild type Zfra (~40% death; n=8), S8G mutant lost its function in causing cell death (~20%; $p<0.001$, n=8; Student's t test). T20G mutant was functionally active in inducing cell death ($p>0.05$, n=8, versus wild type Zfra). Data were not shown for the 24-hr culture. (from ref. #12)

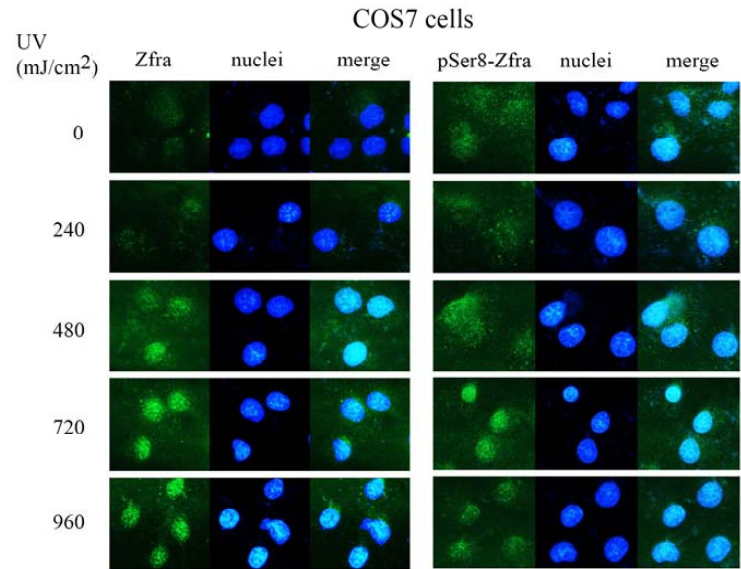
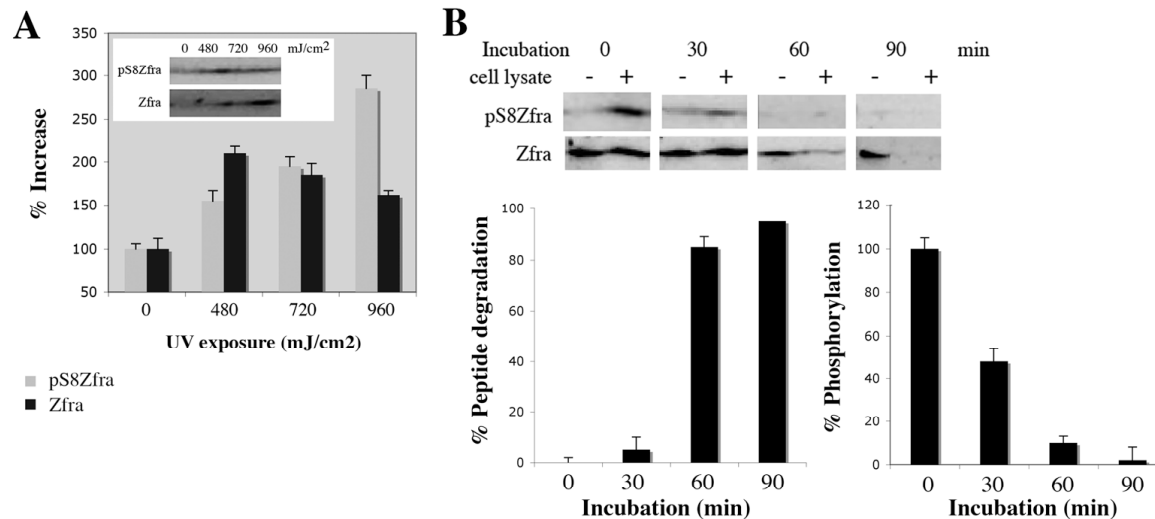


Fig. 5. UV light upregulates Zfra expression, Ser8 phosphorylation and nuclear accumulation. COS7 fibroblasts were exposed to various energy levels of UV light, followed by culturing for 1 hr at 37°C, and then processing immunofluorescence staining using specific antibodies against Zfra and pS8Zfra (phosphorylation at Ser8), respectively (12). Upregulation of Zfra and pS8Zfra expression, along with nuclear accumulation, is shown (adapted from ref. #12).

Fig. 6. Cytosolic kinase(s) induces Zfra phosphorylation at Ser8 followed by degradation. (A) In parallel with the above experiments, UV light increased the expression of Zfra protein and Ser8 phosphorylation, as determined by Western blotting. A bar graph is shown (mean \pm standard error; n=3). (B) A full-length Zfra peptide was synthesized, and aliquots of the peptide (1mM in phosphate-buffered saline) were mixed with the cytosolic fractions of COS7 cells (40 μ g protein) and incubated at 37°C for indicated times. Zfra was rapidly phosphorylated at Ser8 upon mixing with the cytosolic proteins, followed by dephosphorylation and degradation (adapted from ref. #12).



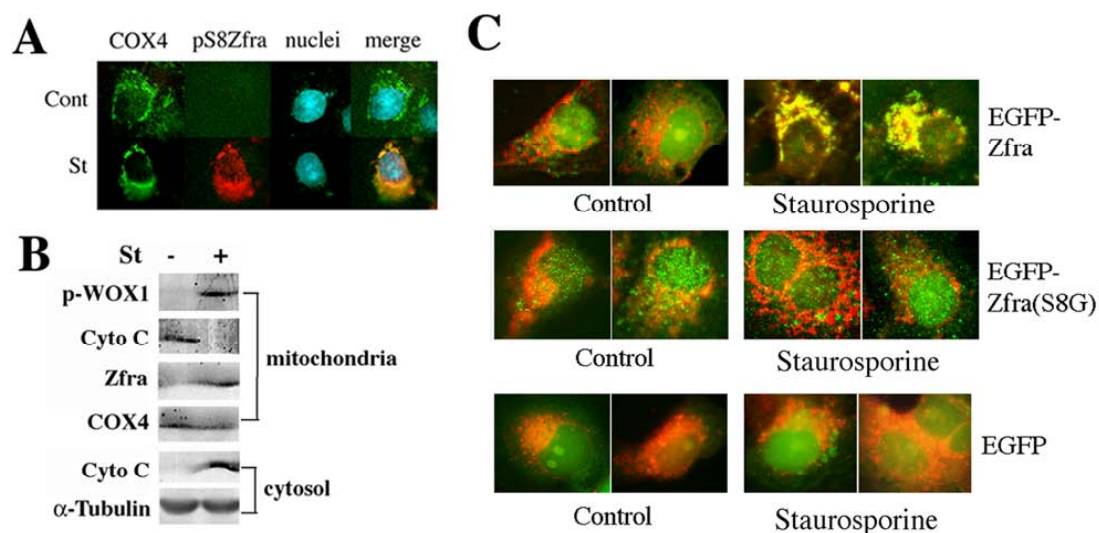
Ser8 phosphorylation is essential for Zfra to relocate to the mitochondria - To examine the role of Zfra during apoptosis, COS7 cells were stimulated with staurosporine for 30 min to induce the mitochondrial pathway of apoptosis. Endogenous Zfra became Ser8-phosphorylated and migrated to the mitochondria (Fig. 7A). Notably, the nuclear level of Ser8-phosphorylated Zfra was low (Fig. 7A). This is in contrast to the effect of UV light in inducing nuclear translocation of Zfra (Fig. 5). Additionally, staurosporine-induced translocation of endogenous Zfra and Tyr33-phosphorylated WOX1 (p-WOX1) to the mitochondria, along with cytochrome c release (Fig. 7B). WOX1 translocates to the mitochondria and nuclei in response to stress stimuli both *in vitro* and *in vivo* (3,4). Under similar conditions, betulinic acid stimulated the opening of mitochondrial transition pores, which allowed translocation of Ser8-phosphorylated Zfra to these organelles (12). Endogenous Zfra binds p-WOX1 when cells are exposed to TNF, UV light or other genotoxic stress (11,33), suggesting that both proteins co-relocate to the mitochondria.

Indeed, Ser8 phosphorylation is essential for Zfra to translocate to the mitochondria. Transient overexpressed EGFP-Zfra is normally localized in the nuclei (Fig. 7C). Stimulation of EGFP-Zfra-expressing COS7 cells with staurosporine induced relocation of the protein to the mitochondria (Fig. 7C; see the overlapped yellow fluorescence). In contrast, S8G-Zfra mutant and EGFP did not translocate to the mitochondria in response to staurosporine (Fig. 7C; see the green fluorescence in the nuclei). Also, S8G-Zfra mutant and EGFP alone did not induce apoptosis (Fig. 4).

Zfra blocks betulinic acid-induced cytochrome c release – COS7 cells were transfected with EGFP, EGFP-Zfra, S8G or T20G mutant, and stimulated with betulinic acid to induce mitochondrial permeability transition (MPT) for releasing cytochrome c. Cytochrome c release to the cytosol

occurred in the control EGFP cells, but was blocked in cells expressing EGFP-Zfra, S8G, or T20G mutant (Fig. 8A). The cytosolic levels of proapoptotic WOX1, isoform WOX2, and p53 were relatively unchanged among these tested cells (Fig. 8A). WOX1 physically interacts with p53 and both proteins induce apoptosis synergistically (3,6,7). Similar results were obtained by stimulating cells with staurosporine (1 μ M for 30 min). For verification, COS7 cells were transfected with ECFP (enhanced cyan fluorescent protein)-Zfra or ECFP alone, cultured 24 hr, and then treated with betulinic acid for 1 hr. Cytochrome c was shown to release to the cytosol in ECFP-expressing cells (Fig. 8B). In contrast, ECFP-Zfra effectively blocked the release (Fig. 8B). Again, similar results were observed with staurosporine (1 μ M for 30 min).

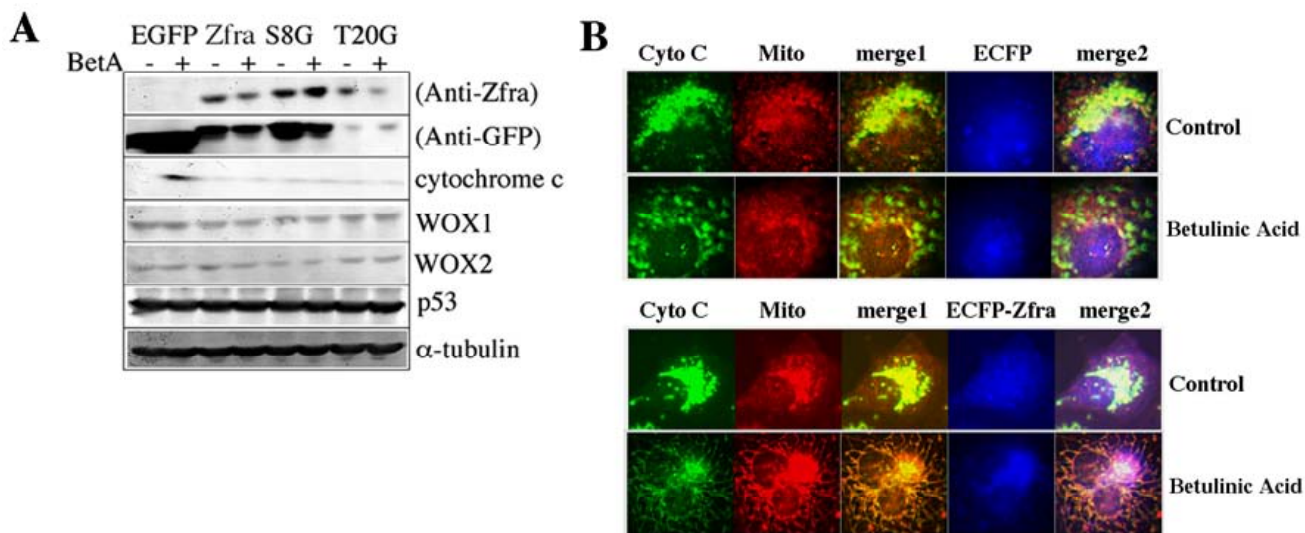
Fig. 7. Ser8 phosphorylation is essential for Zfra translocation to the mitochondria. (A) COS7 cells were stimulated with staurosporine (St; 0.5 μ M) for 30 min, which results in Ser8-phosphorylation of endogenous Zfra and its translocation to the mitochondria (stained with COX4 antibody). COX4, mitochondrial cytochrome c oxidase subunit IV. (B) COS7 cells were stimulated with staurosporine (1 μ M; St) for 30 min, which resulted in translocation of Zfra and Tyr33-phosphorylated WOX1 (p-WOX1) to the mitochondria. Cytochrome c was released to the cytosol and disappeared from the mitochondria. The level of COX4 remained relatively unchanged. Cyto C, cytochrome c. A set of representative data is shown from two experiments. (C) Staurosporine (1 μ M) induced relocation of ectopic EGFP-Zfra (green) from the nuclei to the mitochondria (red), which appears yellow for the colocalized area (yellow). Staurosporine did not induce accumulation of the S8G-Zfra mutant or EGFP alone to the mitochondria (green fluorescence in the nuclei). (adapted from ref. #12)



Zfra prevents WOX1-dependent cytochrome c release - When overexpressed, the proapoptotic WOX1 induces cytochrome c release. COS7 cells were cultured for 48 hr in the presence of retrovirus expressing small interfering RNA targeting WOX1 (WOX1si) (7,10,37), followed by treating with staurosporine to induce cytochrome c release. In controls, empty virus was used to infect cells. WOX1si suppressed approximately 70% of WOX1 protein expression (Fig. 9A). Staurosporine caused cytochrome c release from the mitochondria in cells infected with empty retrovirus (~100 cells examined and approximately 70% with cytochrome c release; Fig. 9A). In contrast, no cytochrome c release was observed in cells expressing WOX1si (~100 cells examined and less than 10% with cytochrome c release; Fig. 9A). We examined whether Zfra blocks WOX1-dependent cytochrome c

release in COS7 cells. Cells were transfected with expression constructs of EGFP-Zfra and/or WOX1 (or EGFP alone) by electroporation, followed by culturing for 48 hr and treating with betulinic acid for 1 hr. By Western blotting, Zfra was shown to inhibit WOX1-dependent cytochrome c release to the cytosol (Fig. 9B). The ectopic WOX1 and Zfra were mainly colocalized in the mitochondria (data not shown).

Fig. 8. Zfra suppresses betulinic acid-induced cytochrome c release. (A) COS7 cells were transfected with the expression construct of EGFP, EGFP-Zfra, S8G, or T20G mutant by electroporation, cultured 24 hr, and then stimulated with betulinic acid (50 μ M; BetA) for 1 hr. Cytochrome c was released to the cytosol in the control EGFP cells, but was blocked in cells expressing Zfra, S8G, or T20G mutant. One set of data is shown from two experiments. (B) COS7 cells were electroporated with ECFP-Zfra or ECFP alone (plasmid), cultured 24 hr, and treated with BetA (50 μ M) for 1 hr. BetA induced cytochrome c release from the mitochondria in ECFP-expressing cells. Mitochondria were stained with mouse antibodies against COX4 (red), and cytochrome c with specific rabbit polyclonal antibodies (green). Presence of cytochrome c in the mitochondria is shown as “yellow” (overlapped areas from red and green). BetA-induced cytochrome c release to the cytosol is shown (see “green” spots in the overlapped picture). Zfra blocked BetA-induced cytochrome c release from the mitochondria (very few or no green spots). Approximately ~100 cells were examined, which showed >90% inhibition of cytochrome c release. (adapted from ref. #12)



Summary – Zfra is a small size 31-amino-acid C2H2 zinc finger-like protein, which is known to interact with JNK1, WOX1, TRADD and NF- κ B during stress response (11,12,33). We have determined that in response to UV light, Zfra becomes Ser8 phosphorylated and translocates mainly to the nuclei (12) (Fig. 10; see Route 1). However, induction of the mitochondrial pathway of apoptosis by staurosporine or betulinic acid, Ser8-phosphorylated Zfra translocates to the mitochondria to induce apoptosis (Fig. 10; Route 2). Alteration of Ser8 abolishes relocation of Zfra to the mitochondria and no cell death occurs. Zfra exhibits an intriguing characteristic in inducing the mitochondrial pathway of apoptosis. Namely, overexpressed Zfra significantly downregulates Bcl-2 and Bcl-xL (Route 2), blocks cytochrome c release from the mitochondria (Route 3), and yet induces dissipation of mitochondrial membrane potential, thus leading to apoptosis (Route 4) (Fig. 10; summarized from ref. #12). This novel finding runs against a general belief that blocking of apoptosis inhibitors Bcl-2 and Bcl-xL facilitates cytochrome c release and causing apoptosis.

A novel mechanism for Zfra inhibition of cytochrome c release is its binding and blocking WOX1 function (Route 4). Overexpressed WOX1 is shown to induce cytochrome c release (12). Finally, Zfra appears to exhibit a plethora of functions in regulating cell growth and death. Mechanistically, how Zfra affects protein and cellular functions is largely unknown and has yet to be determined.

Fig. 9. Zfra blocks WOX1-induced cytochrome c release from the mitochondria. (A) COS7 cells were infected with retrovirus expressing small interfering RNA targeting WOX1 (WOX1si). These cells were cultured for 48 hr, and then treated with staurosporine (1 μ M for 1 hr). In controls, cells were infected with an empty virus. Staurosporine (Stauro) induced cytochrome c release from the mitochondria in control cells (~70% cells), whereas this event did not occur effectively in cells expressing WOX1si (less than 10%). Cytochrome c (green) was stained with specific monoclonal antibodies, and mitochondria with specific antibodies against COX4 (red). (B) COS7 cells were transfected with expression constructs of EGFP-tagged Zfra and/or WOX1 (or EGFP alone) by electroporation, followed by culturing for 48 hr and treating with betulinic acid (50 μ M; BetA) for 1 hr. Cells were harvested and cytosolic fractions were isolated. Zfra inhibited WOX1-dependent cytochrome c release to the cytosol.

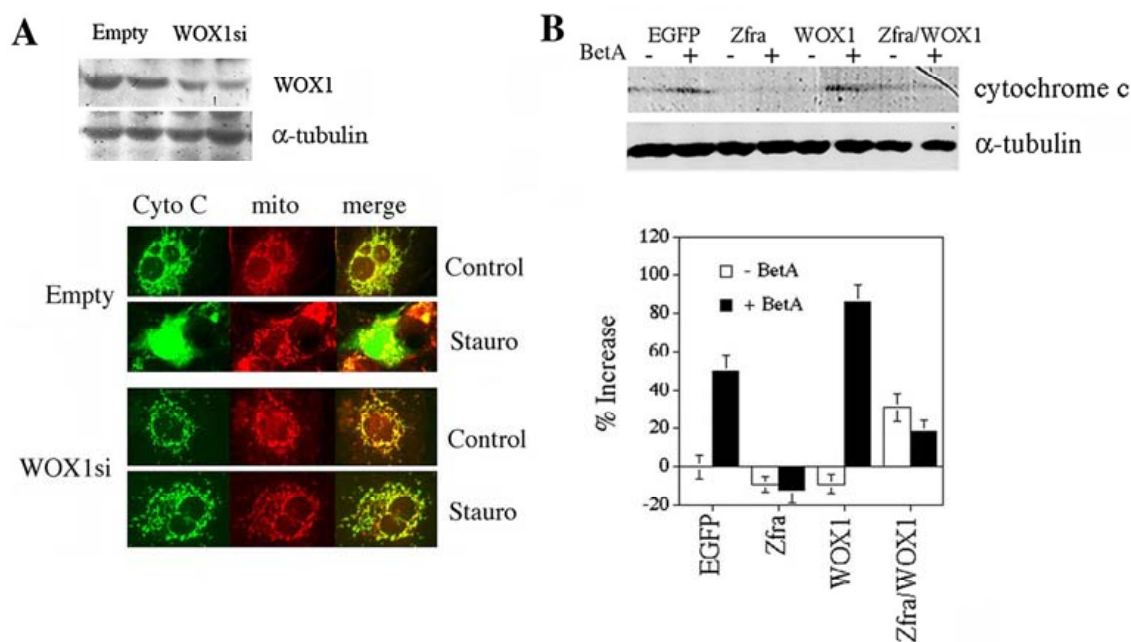


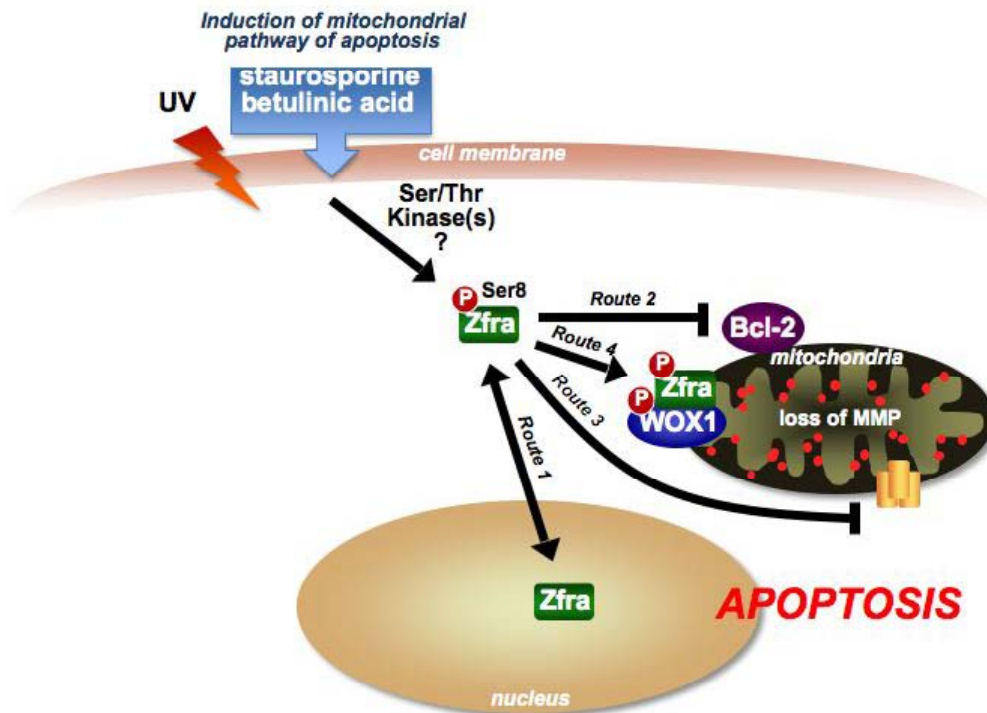
Fig. 10. A schematic model of Zfra action – induction of apoptosis at both nuclear and mitochondrial levels.

Route 1: Zfra becomes Ser8 phosphorylated and translocates mainly to the nuclei in response to UV light.

Route 2: Staurosporine or betulinic acid-induced mitochondrial pathway of apoptosis involves Zfra phosphorylation at Ser8 and relocation to the mitochondria.

Route 2/3: Overexpressed Zfra significantly downregulates Bcl-2/Bcl-xL, blocks cytochrome c release from the mitochondria, and yet induces dissipation of mitochondrial membrane potential.

Route 4: Zfra inhibition of cytochrome c release is due in part to its binding and blocking the WOX1 apoptotic function.



Key Research Accomplishments

Our original proposed **Specific Aims** are:

- 1) Determine how Zfra undergoes covalent self-aggregation and binding with tumor suppressor proteins for regulating their functions. Recombinant p53, WWOX, NF-κB and JNK1 will be mixed with synthetic Zfra for covalent conjugation. Proteomics/mass sequencing analyses will be performed to identify the donor and acceptor amino acids in Zfra and protein targets, respectively. Apoptotic stimuli (e.g. tumor necrosis factor) will be used to control zfration/de-zfration, versus protein phosphorylation, by using recombinant and endogenous proteins.
- 2) Determine the overall protein targets with which Zfra interacts. Synthetic Zfra will be covalently conjugated with whole cell proteins, followed by immunoaffinity purification and mass sequencing. The identified proteins will be assessed by computational analyses and chemical modeling regarding Zfra-induced functional inactivation.
- 3) Investigate the effect of Zfra deficiency in breast cancer cells. Breast cancer cells, either metastatic or non-metastatic, will be reinstated with ectopic Zfra gene. Growth of these cells in culture and in nude mice will be tested, thereby providing insight into Zfra regulation of breast cancer cells.

Promising findings from our published work have demonstrated that Zfra regulates TNF signaling via interactions with receptor adaptor proteins and downstream effectors, including TRADD, FADD, RIP, JNK1, p53, and WOX1 (11,12,33). During the mitochondrial pathway of apoptosis, Zfra becomes phosphorylated at Ser8 and relocates to the mitochondria, and may block the apoptotic function of tumor suppressor WWOX and p53 in these organelles (12).

During the past one-funding year, our **key research discoveries** thus far are: 1) enhancement of degradation of zfrated tumor suppressor WWOX and other proteins independently of the ubiquitination/proteasome system, 2) enzyme-independent self-polymerization of Zfra for more than 200 fold, and 3) Zfra peptide enhancement of the growth of skin basal cell carcinoma in nude mice, suggestive of its inactivation of tumor suppressors *in vivo*.

Specific Aim 1: Zfra self-aggregation and functional inactivation of proteins

Zfra is a 31-amino-acid protein possessing 5 conserved phosphorylation sites, where Ser8 phosphorylation is essential for Zfra to relocate to the mitochondria and induce apoptosis (11,12). The amino acid sequence of Zfra is: MSSRRSSSCK YCEQDFRAHT QKNAATPFLA N. Not all cells are sensitive to overexpressed Zfra-mediated cell death. For example, prostate DU145 and normal mink lung epithelial Mv1Lu cells are resistant to Zfra-mediated cell death (12). Zfra is short but similar to the family of C2H2 type zinc finger proteins, which may bind DNA and RNA (38). Zfra is able to “zip” or undergo self-association from the interactions between its cysteines (Cys 9 and 12). Nonetheless, this type of binding is subjected to dissociation under reducing conditions. Evidence shown below is the occurrence of covalent self-aggregation of Zfra in the presence of phosphate. The covalent interactions involve not only cysteines but also other unidentified residues, as determined by comparing reducing and non-reducing SDS-PAGE.

E3 ubiquitin ligase mediates covalent attachment of ubiquitin to a lysine on a target protein via an isopeptide bond (36). Polyubiquitination occurs when a second ubiquitin is attached to the first, a third to the second, and so forth. Polyubiquitinated proteins are subjected to degradation by the proteasome system. In contrast, mono-ubiquitination in proteins is mainly for functional maturation or alteration, but not targeted for proteasomal degradation. Sumoylation often occurs on a specific lysine residue within the consensus motif ψ KxE/D, where ψ is a hydrophobic amino acid, x is any amino acid, and K is the site of SUMO conjugation (39). Whether K10 and K22 in Zfra participate in self-polymerization is unknown.

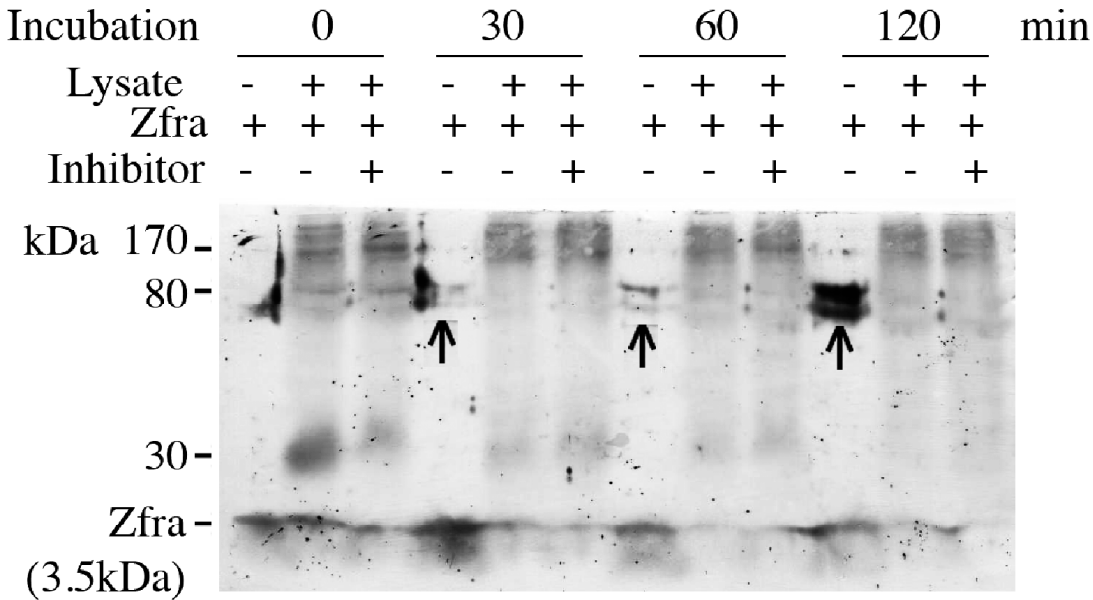
Zfra self-polymerizes independently of catalytic enzymes and binds cellular proteins covalently – Full-length Zfra peptide was synthesized (>98% pure) by the core facility at the University of Texas San Antonio and Genemed Synthesis (San Antonio, Tx), respectively. In MilliQ water, Zfra goes to solution (at 2 mM). Interestingly, upon exposure to phosphate-buffered saline (PBS), Zfra rapidly went out of solution and became visible amorphous precipitates. Incubation of Zfra in PBS for longer than 1 hr at room temperature or at 37°C resulted in two protein bands of ~78 and 80 kDa (polymerized from a 3.5 kDa unit), as determined under reducing SDS-PAGE (Fig. 11; see arrows). Under non-reducing gel electrophoresis, the sizes of Zfra are greater than 158 kDa (data not shown). The observations indicate that Zfra monomers interact with each other via cysteines and other residues. Importantly, these reactions are self-catalytic and enzyme independent.

We examined whether Zfra interacts with cellular proteins in Zfra-negative cells. Lysates of Zfra-deficient breast MCF7 cells (33) were prepared and incubated with the full-length Zfra peptide, in the presence or absence of a proteasome inhibitor zLLL (Z-Leu-Leu-Leu-aldehyde) or MG-132 (40 μ M). The results showed the presence of complex formation of Zfra with cellular proteins (or zfrated proteins), and the protein complexes degraded with time of incubation at 37°C. zLLL did not block the degradation, indicating that the ubiquitin/proteasome system is not involved in the degradation process (Fig. 11). Indeed, presence of zLLL rapidly enhanced the degradation of a zfrated 30-kDa protein (Fig. 11; see time

0).

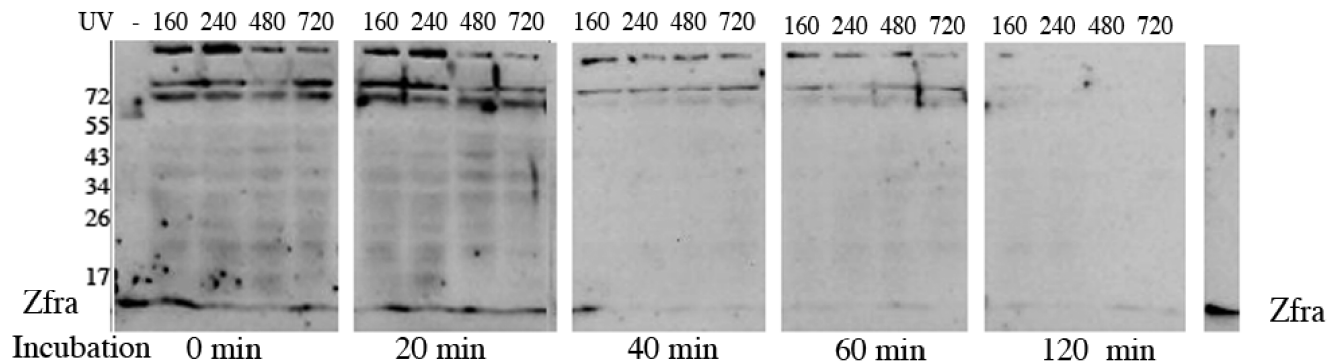
Similarly, Zfra-deficient prostate Du145 cells were exposed to UV light (mJoule/cm²) and incubated for 30 min prior to preparing whole cell lysates. Incubation of the cell lysates with the synthetic Zfra peptide rapidly resulted in covalent conjugation of Zfra with cellular proteins (Fig. 12). Again, Zfra was not dissociated from the target proteins under reducing SDS-PAGE (or resistant to dissociation by β -mercaptoethanol and SDS). The protein complexes disappeared with time of incubation, indicating that degradation of these complexes occurred. Together, the above observations indicate that zfrated proteins are subjected to degradation independently of the ubiquitination/proteasome system.

Fig. 11. Zfra covalently binds cytosolic proteins in MCF7 cell lysates. First, upon exposure to PBS, the synthetic Zfra peptide alone formed covalent complexes by itself (78 and 80 kDa) after incubation for 30-120 min at 37°C, as determined by Western blotting using specific Zfra antibody (see **arrows**). The SDS-PAGE was run under reducing conditions. Second, cell lysates were prepared from Zfra-deficient MCF7 cells. Aliquots of the lysates (100 μ g) were incubated with Zfra peptide (100 μ M) for various durations in the presence of PBS and/or proteasome inhibitor zLLL or MG-132 (50 μ M). Formation of covalent complexes is shown (170, 80, 30 kDa and many minor bands) under reducing SDS-PAGE. Synthetic full-length Zfra is 3.5 kDa. The complexes were degraded with time of incubation even in the presence of zLLL. Presence of zLLL rapidly enhanced the degradation of a zfrated 30-kDa protein (Fig. 11; see time 0).



Potential role of Tyr33 in WOX1 for zfration: development of zfration assay – Indeed, we have generated promising data, which may quickly lead to identification of protein zfration sites. We have mapped the binding of Zfra to the *N*-terminal first WW domain and *C*-terminal SDR domain of WOX1 by yeast two-hybrid analysis (Fig. 14) (11). Alteration of Tyr33 to Arg33 in the first WW domain abolished the binding of Zfra to WOX1, suggesting that Tyr33, when phosphorylated, may cross-link with one of the serines (e.g. Ser8) in Zfra. By FRET, we have confirmed the binding of Zfra to the *N*-terminal first WW domain with Tyr33 phosphorylation (Lee *et al.*, submitted). Thus, based on the amino acid sequence of WOX1 at the Tyr33 area, we may be able to determine the likely presence of a structural motif in proteins for zfration from protein databases.

Fig. 12. Zfra covalently interacts with cellular proteins. Du145 cells do not express Zfra. The cells were exposed to UV (mJ/cm^2) and whole lysates ($100\ \mu\text{g}$) were prepared and incubated with Zfra peptide ($100\ \mu\text{M}$) for various durations. Formation of covalent complexes is shown (70, 72, 200 kDa and many minor band ladders) under reducing SDS-PAGE. Synthetic full-length Zfra is 3.5 kDa. The complexes were degraded with time of incubation.



Determine the specific site(s) in WOX1 for Zfra to interact in a covalent manner – By co-immunoprecipitation, here we showed that WOX1 undergoes zfration. Zfra-expressing COS7 fibroblasts were exposed to UV light ($480\ \text{mJoule}/\text{cm}^2$), followed by culturing for 30 min at 37°C and then processing co-immunoprecipitation. The precipitated WOX1 was Tyr33-phosphorylated and zfrated as determined using specific antibody against Zfra (Fig. 13). It appeared that there was a reduced zfration in WOX1. We will determine in time-course and dose-related experiments for establishing the event of zfration/de-zfration under apoptotic or stress conditions.

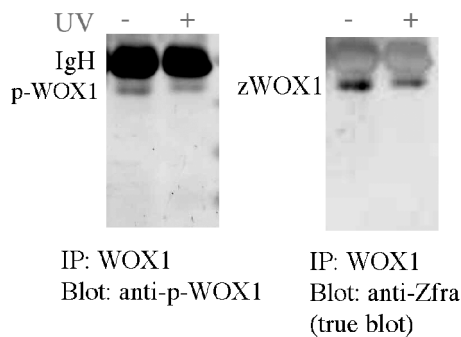


Fig. 13. Zfration in WOX1. Zfra-expressing COS7 fibroblasts were exposed to UV light ($480\ \text{mJoule}/\text{cm}^2$), followed by culturing for 30 min at 37°C , and then processing co-immunoprecipitation. The precipitated WOX1 was both Tyr33-phosphorylated and zfrated, as determined using specific antibody against Zfra. True blot: a commercial antibody barely recognized IgG (eBioscience).

Specific Aim 2: Determine the overall protein targets with which Zfra interacts

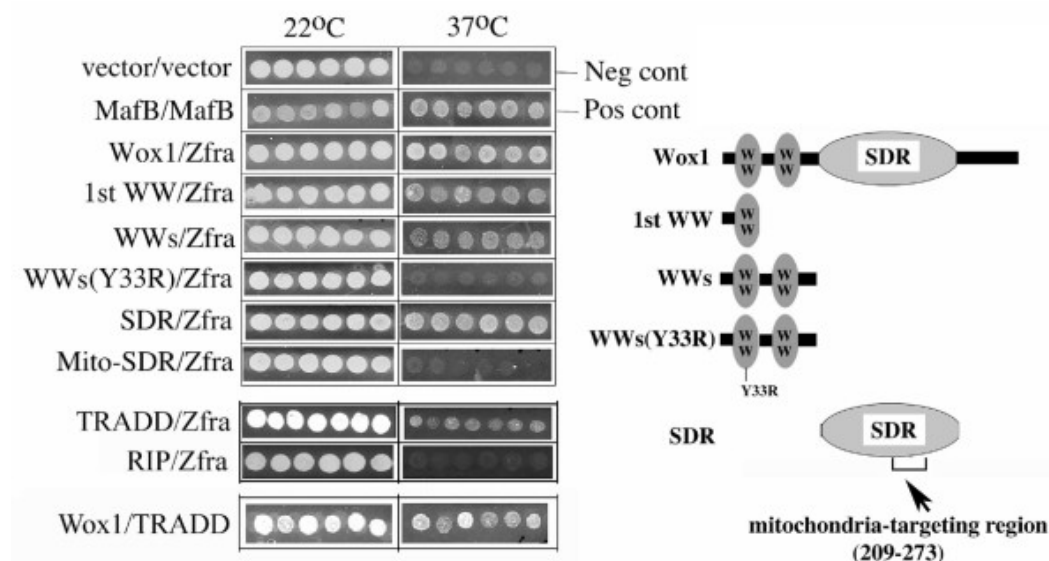
This Aim involves conjugation of synthetic Zfra with whole cell proteins, followed by immunoaffinity purification and mass sequencing. The identified proteins have been and will be assessed by computational analyses and chemical modeling regarding Zfra-induced functional inactivation. Preliminary work has shown that Zfra interacts covalently with actin as a major protein. Nonetheless, there are thousands of proteins interacting with Zfra. We are still sorting out our results and hope to find a common trend regarding Zfra-binding proteins.

Specific Aim 3: effect of Zfra in breast cancer development

Exogenous Zfra enhances growth of skin basal cell carcinoma (BCC) in nude mice – In the preliminary experiments, we tested our **hypothesis** that zfrated tumor suppressor proteins are functionally inactivated, and that de-zfration allows regain of their function for tumor suppression. In a xenograft tumor model, nude mice were inoculated with BCC and then received intratumoral injection of purified Zfra peptide. The peptide did not shrink the tumors. Instead, the tumor sizes were 3-4 fold larger than controls in 3 months (Fig. 15; a representative data from 3 experiments). Similar results were observed by testing the growth of breast MDA-MB231 cells in nude mice (i.e. Zfra caused 2-3 fold increases in tumor volume).

We believe that blocking of tumor suppressors by Zfra enhances cancer growth. These observations are in agreement with our *in vitro* findings that Zfra blocks the function of tumor suppressors p53 and WOX1 (11,12), and further support our **hypothesis** that Zfra is a global inhibitor of tumor suppressor proteins.

Fig. 14. Zfra physically binds TRADD and the N-terminal first WW domain and C-terminal SDR domain in WOX1. Binding of Zfra (as target) with WOX1 (as bait) was mapped by Ras rescue-based yeast two-hybrid analysis (11; see the article in the attachment). Positive binding is evidenced by the growth of mutant yeast at 37°C in a selective medium. Zfra bound to the N-terminal first WW domain and the C-terminal SDR domain of WOX1. Alteration of the known phosphorylation site Tyr33 to Arg33 (Y33R) in WOX1 abrogated the binding. Zfra did not interact with the mitochondria-targeting region in the SDR domain (Mito-SDR). No yeast cell growth at 37°C was shown when empty vector versus empty vector was tested as a negative control. In a positive control, the self-binding of MafB is shown. In addition, Zfra was shown to bind the full-length TRADD, but not RIP. WOX1 also bound TRADD. Neg cont: negative control; Pos cont: positive control. (adapted from ref. #11)

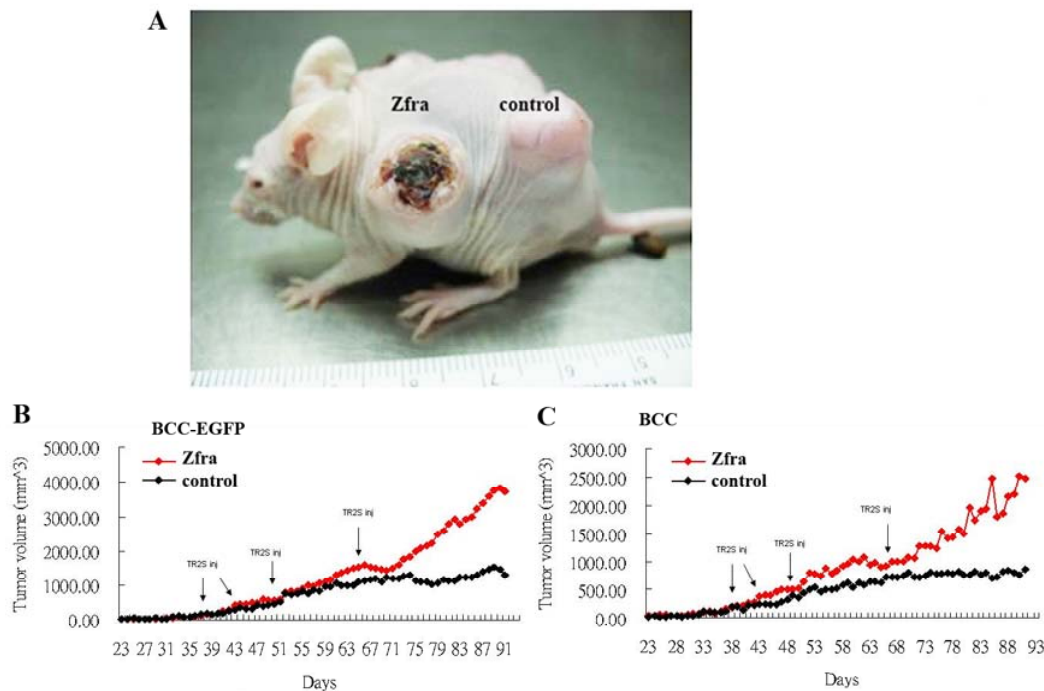


In comparative experiments, the Ac-Zfra peptide (acetylated at K10 and K22) exhibited similar functions in increasing the tumor sizes as that of the wild type Zfra peptide (2-4 fold increases in 3 months). Again, the observations suggest that critical amino acid, other than K10 and K22, are involved in zfration

of tumor suppressor proteins for functional inactivation.

High speed imaging for WOX1 zfraction/de-zfraction on cell surface – Zfraction/de-zfraction is probably a critical event in regulating cancer cell growth. We have been and will carry out imaging of zfraction by confocal fluorescence microscopy. Zfra peptide undergoes self-aggregation rapidly upon exposure to phosphates. This event may occur in millisecond or even nanoseconds. Interestingly, aggregated Zfra (or poly-Zfraction) exhibits strong autofluorescence (most strong in red and green) (data not shown). In similar experiments, seeding of breast MCF7 cells in the presence of the full-length peptide, followed by culturing for 24-48 hr, resulted in formation of cell aggregation or colonies (Fig. 16). The cell colonies exhibited red and green autofluorescence. It appears that Zfra aggregates were deposited on the cell surface or internalized by the cells, and that these cells grew in a colony-like manner. The autofluorescence may be regarded as a marker for zfraction. Particularly, when a protein is poly-zfractioned, there is a good chance of exhibiting autofluorescence.

Fig. 15. Zfra enhances the growth of human skin basal cell carcinoma (BCC) in nude mice. Nude mice were inoculated with BCC expressing EGFP or nothing. (A,B) 10 μ l of Zfra peptide (2 mM in sterile Milli-Q water) was injected into a BCC solid tumor expressing EGFP (enhanced green fluorescent protein). The date of injection is shown (see arrows). Control injection was 10 μ l sterile water only. (C) Similarly, Zfra peptide or sterile water was injected onto a BBC tumor (without ectopic EGFP). A representative data is shown from 3 experiments.



We have recently shown the presence of WOX1 on the cell membrane as a protein complex with hyaluronidase Hyal-2 (29). We determined this by immunoelectron microscopy (29) and by TIRF (Total Internal Reflection Fluorescence) microscopy (40). For example, complement C1q induced morphological changes of cell surface by generating thousands or millions of microvilli mainly on or

between DU145 cells, and that WOX1 is present on the microvilli (40). C1q broke down these microvilli into “punctates” with time.

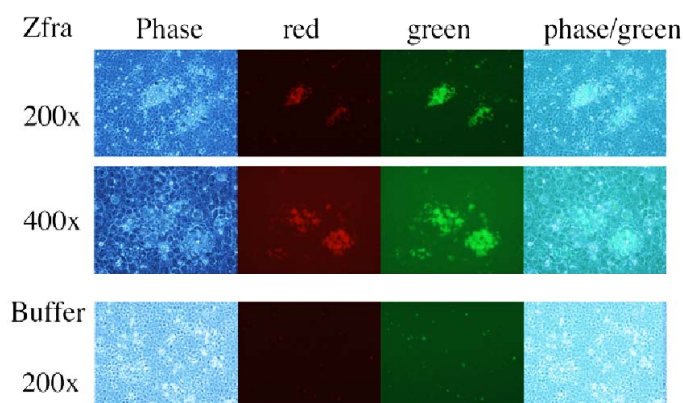


Fig. 16. Co-culture of breast MCF7 cells with Zfra peptide. MCF7 cells were seeded onto Petri dishes in the presence or absence of Zfra peptide (PBS buffer alone as a control). 24-48 hr later, the cells showed formation of colonies with Zfra aggregates deposited on or inside the cells. The cells have a strong autofluorescence for red and green.

Reportable Outcomes

We are preparing two manuscripts for publications as a result of the DoD funding. Briefly, in the first article, we will address our major discovery that 1) Zfra undergoes enzyme-independent self-polymerization for more than 200 fold, and 2) Zfra enhances of degradation of zfrated tumor suppressor WOX1 and other proteins independently of the ubiquitination/proteasome system. In the second article, we will describe the finding that Zfra peptide enhances of the growth of many types of cancer cells, including breast, brain, skin, and others.

Publications supported in part by the DoD funding are:

- 1) Hsu LJ, Schultz L, Hong Q, Van Moer K, Heath J, Li MY, Lai FJ, Lin SR, Lee MH, Lo CP, Lin YS, Chen ST, **Chang NS** (2009) Transforming growth factor beta1 signaling via interaction with cell surface Hyal-2 and recruitment of WWOX/WOX1. *J Biol Chem.* **284**, 16049-16059.
- 2) Hong Q, Sze CI, Lin SR, Lee MH, He RY, Schultz L, Chang JY, Chen SJ, Boackle RJ, Hsu LJ, **Chang NS** (2009) Complement C1q activates tumor suppressor WWOX to induce apoptosis in prostate cancer cells. *PLoS One* **4**, e5755.
- 3) Li MY, Lai FJ, Hsu LJ, Lo CP, Cheng CL, Lin SR, Lee MH, Chang JY, Subhan D, Tsai MS, Sze CI, Pugazhenth S, **Chang NS***, Chen ST (2009) Dramatic co-activation of WWOX/WOX1 with CREB and NF- κ B in delayed loss of small dorsal root ganglion neurons upon sciatic nerve transection in rats. *PLoS ONE*. in press. (*corresponding author)

Conclusion

In this funded research, we have described “zfration” - a naturally occurring small-size peptide Zfra undergoes self-covalent binding and aggregation, and may covalently interact with tumor suppressors for functional inactivation. We determined that Zfra can 1) induce degradation of intracellular zfrated proteins, 2) undergo enzyme-independent self-polymerization for more than 200 fold of its original size, and 3) significantly increase the growth of breast and skin basal cell carcinoma in nude mice by 3-4 fold in 3 months, suggestive of its inactivation of tumor suppressors *in vivo*.

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